CLXXXIV. THE COAGULATION AND INACTIVATION OF EMULSIN BY HEAT: INFLUENCES OF CRYSTALLOIDAL AND OF COLLOIDAL SOLUTES.

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It was shown by Hanzlik and Butt [1928] that Congo red and certain other colloidal dyes can prevent death in animals injected with otherwise fatal doses of drugs and toxins. Hanzlik [1932] also showed that a certain series of dyes, *viz.* Congo red, vital red, trypan red, mercurochrome, rose Bengal and sodium fluorescein prevented the coagulation *in vitro* of whole serum (horse) or egg albumin on heating to boiling or adding mercuric chloride. Certain other colloids, colloidal dyes, and crystalloidal dyes listed by Hanzlik did not prevent heat coagulation of serum and egg albumin under the same conditions.

The demonstration of the protective action of Congo red and certain other dyes upon egg albumin and tissue extracts was repeated by one of us (D. L. F.) with the same general results as those reported by Hanzlik. Since the addition of certain substances to colloidal systems prevents the radical changes in structure and dispersion which otherwise attend heating, it was thought profitable to investigate the question of whether the function of a colloiddally dispersed protein-containing enzyme could be preserved, in part at least, by adding similar agents to its solutions before heating. Statements have been made to the effect that various anticoagulating agents do not prevent the inactivation of enzymes by various kinds of treatment. Ter Meulen [1905] found that emulsin was protected at 51° by both glucose and lactose, and inhibited only by the former. Waldschmidt-Leitz [1929] cites the work of numerous investigators who studied the inactivation of enzymes, notably Euler and his co-workers who investigated the inactivation of a saccharase by mercuric chloride and the regeneration of the activity by the precipitation of the mercuric ion by hydrogen sulphide. Sucrose was shown to protect saccharase against poisoning by mercuric ion, but not by silver ion. Euler and Myrbäck inactivated saccharase with organic bases and annulled this inactivation by aldehydes, which led to the conclusion that the original inactivation of the saccharase was due to the formation of some kind of aldehyde-amine compound. Haas and Hill [1928] cite the work of several different investigators who studied the restoration to active function of enzymes which had been inactivated by heat; thus Falk brought about the partial recovery of lipase action by the addition of manganese; Biedermann discovered that vigorous shaking with air restored the activity of salivary amylase which had been heated to 100°, whilst Gallagher found that heating the peroxidase of the mangold at 100° for a short interval temporarily destroyed the function of the enzyme, but that it recovered its properties on standing for a time in the cold; the addition of iron and an aldehyde, both of which occurred

normally in the enzyme preparation, was observed to increase the activity of the peroxidase. Space does not permit further discussion here of the numerous experiments upon the inactivation of enzymes by various workers.

In the present work attention was devoted to the possible influences of the hydrogen ion, certain colloidal dyes and two sugars, upon the threshold of heat inactivation of emulsin¹. In this problem, we have not paid attention to relative rates of inactivation of emulsin at different temperatures and under different conditions, nor have we been concerned with the measurement of the quantity of HCN produced from the hydrolysis of amygdalin by emulsin treated in various ways. We merely measured, as closely as possible by repeated trials, the temperature above which a controlled quantity and concentration of emulsin was inactivated by 30 minutes of incubation. The test for residual enzymic function following heat treatment was made by adding a definite quantity of the substrate, amygdalin, in the same concentration throughout all the experiments, and then incubating the mixture at a moderate temperature (39°), maintaining in the system a sensitive indicator to reveal the formation of any HCN. Two different intervals were employed for this test: 1 hour at 39°, and overnight in the water-bath which was allowed to cool to room temperature during that interval. The substrate employed throughout the work was pure amygdalin². This particular system was employed because there was an obvious advantage in being able to test for HCN, one of the products of hydrolysis, in the air, over the colloidal and usually highly coloured liquid, and thus to determine whether or not the enzyme was still active.

A solution of 1 % amygdalin in boiled distilled water was prepared and kept under toluene as a preservative. A similar solution of 1 % emulsin was prepared, filtered through paper and stored in like manner. The amygdalin solution was shown to remain undecomposed and the emulsin solution to retain its enzymic function for an indefinite period (*i.e.* after several months).

In all cases experiments were conducted in duplicate and repeated if necessary, using mixtures of 1 ml. emulsin solution with 1 ml. of the solution whose protective action was being investigated. In control duplicates 1 ml. of distilled water was added in each case instead of the second solution. The respective volumes were measured by pipette into glass-stoppered cylinders of 10 ml. or 25 ml. capacity, thoroughly mixed, placed in a glycerol-bath, maintained at constant temperature inside a thermostatically controlled electric oven and incubated there for 30 minutes. At the end of this interval the tubes were removed and cooled to 39° by immersion in a water-bath maintained at that temperature by thermostatic control. Then 1 ml. of 1 % amygdalin + 1 ml. of distilled water at 39° were added to the experimental series of incubated emulsin tubes, and thorough mixing was brought about by rapid swirling. The final volume was thus always brought to 4 ml. For the determination of the temperatures at which unprotected emulsin alone was inactivated in the 30-minute pre-heating interval, 1 ml. of 1 % emulsin solution + 1 ml. distilled water only were mixed and subjected in duplicate to the incubation, after which 1 ml. of 1 % amygdalin solution + 1 ml. distilled water at 39° were added as usual. For the regular controls, always conducted along with the experimental runs, provision was made to ascertain whether or not a particular substance which was being investigated for protective capacity exerted any effects upon the hydrolysis of amygdalin itself, irrespective of the previous history of the emulsin: the emulsin was heated with an equal volume of distilled water, the other substances being added at the same time as was the amygdalin, after the incubation period.

¹ Eimer and Amend. C. P.

² Coleman and Bell, H. P.

After the addition of the amygdalin solution, the activity of the enzyme was tested for by suspending over the fluid in each tube a strip of alkaline sodium picrate paper (Guignard's test), care being taken that the papers were dry enough to allow no transfer of picrate to the solution below. Guignard's test is, under these circumstances, specific for HCN, which reduces the picrate to picramate, causing the paper to change in colour from lemon-yellow, through orange, to brick-red. For confirmation, use can be made of the silver iodide test developed by one of us [Fox, 1934]. Guignard's test was shown by us to possess a sensitivity of 1 part HCN in 200,000 in a period of 1 hour, and of 1:4,000,000

Table I. *The influences of different solutions upon the temperature of heat inactivation of emulsin.*

Exp.	Solution added to emulsin before heating	Solution added to emulsin-amygdalin system for enzyme test	Highest temperature not completely inactivating enzyme in 30 mins.		Precipitation	
			As determined in 1 hour test	As determined in overnight test	When enzyme alone heated	When substrate added and whole incubated at 39°
A	Dist. H ₂ O (control)	Dist. H ₂ O (control)	73°	75°	—	—
R	HCl to give p_H 1.97	Dist. H ₂ O	65.5	70	+ immediately upon mixing	—
A ₁₆	Dist. H ₂ O (control)	HCl → p_H 1.97	73	75	—	+
Q	HCl → p_H 2.97	Dist. H ₂ O	73	75	+ (slight)	—
A ₁₅	Dist. H ₂ O (control)	HCl → p_H 2.97	73	75	—	+ (slight)
L	Phosph. buffer p_H 4.52	Dist. H ₂ O	72-72.5	76	+	—
A ₁₀	Dist. H ₂ O (control)	Phosph. buffer p_H 4.52	72-72.5	76	—	+
N	Phosph. buffer p_H 6.0	Dist. H ₂ O	72-73	75	+	—
A ₁₃	Dist. H ₂ O (control)	Phosph. buffer p_H 6.0	73	75	—	+
M	Phosph. buffer p_H 6.6	Dist. H ₂ O	70-71.5	75	+ above 74°	—
A ₁₁	Dist. H ₂ O (control)	Phosph. buffer p_H 6.6	70-72	75	—	—
O	Phosph. buffer p_H 7.65	Dist. H ₂ O	62	72	+	—
A ₁₃	Dist. H ₂ O (control)	Phosph. buffer p_H 7.65	70.5	75	—	—
P	Phosph. buffer p_H 8.30	Dist. H ₂ O	59	69	+	—
A ₁₄	Dist. H ₂ O (control)	Phosph. buffer p_H 8.30	71	75	—	—
D	1 % neutral red	Dist. H ₂ O	70	70.5-71.5	—	—
A ₂	Dist. H ₂ O (control)	1 % neutral red	70.5-71.5	70.5-71.5	—	—
E	1 % fluorescein	Dist. H ₂ O	62-63	67	—	—
A ₃	Dist. H ₂ O (control)	1 % fluorescein	71.2-73.2	74.5-75.8	—	—
K	1 % rose Bengal	Dist. H ₂ O	{ Increased sensitivity to heat and inhibited the hydrolytic reactions		—	+
A ₉	Dist. H ₂ O (control)	1 % rose Bengal			—	+
B	1 % Congo red	Dist. H ₂ O	65-66.8	72	—	—
A ₁	Dist. H ₂ O (control)	1 % Congo red	73	75	—	—
F	0.5 % Congo red	Dist. H ₂ O	68-69.1	72-73.5	—	—
A ₄	Dist. H ₂ O (control)	0.5 % Congo red	73	74.5-75	—	—
G	0.01 % Congo red	Dist. H ₂ O	72.2-72.5	75	—	—
A ₅	Dist. H ₂ O (control)	0.01 % Congo red	72.2-72.5	75	—	—
H	0.001 % Congo red	Dist. H ₂ O	73	76	—	+ (overnight only)
A ₆	Dist. H ₂ O (control)	0.001 % Congo red	73	76	—	+ (overnight only)
I	0.0005 % Congo red	Dist. H ₂ O	73	76	+ (at 75 or above)	—
A ₇	Dist. H ₂ O (control)	0.0005 % Congo red	73	75-76	—	+ (after heating to 75° +)
J	1 % lactose	Dist. H ₂ O	ca. 73	ca. 75	—	—
A ₈	Dist. H ₂ O (control)	1 % lactose	ca. 73	ca. 75	—	+ (in hour period)
S	50 % glucose	Dist. H ₂ O	75	80	+ (slight)	—
A ₁₇	Dist. H ₂ O (control)	50 % glucose	71	75	—	+ (slight)
T	25 % glucose	Dist. H ₂ O	74	80	+ (slight)	—
A ₁₈	Dist. H ₂ O (control)	25 % glucose	73	75	—	+ (slight)
U	10 % glucose	Dist. H ₂ O	74	78.5	+ (slight)	—
A ₁₉	Dist. H ₂ O (control)	10 % glucose	73	75 (+)	—	— (slight)
V	5 % glucose	Dist. H ₂ O	74	78 (+)	+ (slight)	—
A ₂₀	Dist. H ₂ O (control)	5 % glucose	ca. 73	75 (+)	—	+ (slight)

in an overnight test. The silver iodide test is simple, absolutely specific, and possesses a sensitivity of about 1:2,000,000.

The main experimental results are incorporated in Table I. Since there was always a short lag in the reattainment of the temperature of the glycerol-bath after the glass cylinders containing the enzyme solutions were placed in it, the 30-minute incubation period was seldom at exactly one unvarying temperature. However, the initial drop and subsequent rise were seldom much greater than 1.5°, and sufficient repetitions were performed to allow such overlapping as to indicate closely the true threshold. The temperatures in the table are therefore very close to the true threshold values, although departures of less than 1° should hardly be regarded as significant. The values of 73° as the upper temperature limit at which 30-minute incubation would permit a subsequent positive test for activity in 1 hour, and 75° as the temperature limit as indicated by a subsequent overnight test, were obtained in repeated checks.

The series of incubations in media of different p_H values ranging from 1.97 to 8.3 was conducted originally to determine whether small but unsuspected p_H factors affecting any of the protective experiments would seriously vitiate the results. It is to be noted that only at the low p_H of 1.97 and at the alkaline values does incubation of the enzyme significantly decrease the inactivation temperature threshold. The optimum range of p_H values under the conditions of the experiment were thus from about 3.0 to 6.0. The various dyes and sugar solutions did not, as far as could be determined, impart an alkaline reaction to the water in which they were dissolved. The p_H of the emulsin solution and of the distilled water that was used in preparing the solution was close to 6.0. Whilst all the solutions in both experiments and controls in the p_H series precipitated to different degrees the incubated enzyme, presumably owing to the presence of the electrolytes, it is of interest to note that at p_H values of from 6.6 up, the controls (wherein the emulsin was incubated in distilled water, the buffer being added only at the time that the amygdalin was introduced for the activity test) showed no precipitates, the amygdalin presumably protecting the enzyme from precipitation at the higher p_H values during the test.

Congo red, a colloidal dye which prevents the coagulation of heated egg albumin solutions, did not raise the inactivation temperature of emulsin, but actually lowered it considerably when solutions of 1 % of the dye were added to the enzyme solution before the heating. As the concentration of the added dye was successively decreased, the degree of lowering of the inactivation temperature of the enzyme was steadily decreased in proportion; the presence of 0.001 % of the dye exerted perhaps a slight protective influence. None of the Congo red solutions affected the hydrolysis tests in control experiments.

Fluorescein and rose Bengal, colloidal dyes which also prevent the coagulation of heated solutions of egg albumin, not only failed to protect the enzyme against inactivation by heat, but increased its sensitivity, so that the inactivation temperatures with either dye present were considerably lower. Both of these dyes also exerted inhibitory influences on hydrolysis in the controls. The same was found to be true of neutral red, a dye which does not prevent the heat coagulation of albumin.

The colloidal dyes Congo red, fluorescein and rose Bengal, as would be expected, produced no HCN from amygdalin when mixtures of the glucoside and the respective dyes were incubated for several days.

Lactose at a concentration of 1 % exerted no measurable effects upon the temperature of inactivation, nor did it interfere with hydrolysis. Higher con-

centrations of lactose were not employed since this sugar, being a β -glucoside, is hydrolysed by emulsin, and yields glucose as one of the products.

Glucose, one of the by-products of the hydrolysis of amygdalin, was observed to exert a certain degree of protective action at various concentrations, causing tolerance toward slightly higher temperatures of incubation. The highest concentration of glucose employed exerted a slight retarding effect upon the hydrolysis of amygdalin in the controls, heated in the absence of the sugar. This is taken to signify that solutions of the enzyme heated to the usual threshold of 73° possess insufficient active enzyme to bring about perceptible hydrolysis in the 1 hour period, in the presence of this concentration of glucose, thus accentuating the protective influence of the sugar upon the enzyme heated in its presence. Whether or not the glucose exerted its protective action by virtue of its aldehydic properties was not ascertained.

In general, electrolytes or non-electrolytes, present in true solution, brought about varying degrees of precipitation of the enzyme when this was incubated in their presence. Amygdalin prevented any precipitation of the enzyme in control experiments at p_H values from 6.6 to 8.3, but not in the range from p_H 6.0 down to p_H 1.97. The enzyme alone in distilled water was not precipitated from solution at the inactivation temperatures, nor were enzyme solutions in the presence of the dyes so precipitated, with two exceptions: rose Bengal in final concentrations of 0.25 % brought about precipitation even in the presence of amygdalin, and the weakest solutions of Congo red (final concentration 0.00025 % or below) brought about the same results. These precipitations occurred probably as a result of mutual neutralisation of colloidal charges near the range of sensitisation of the dye.

While the lactose and glucose solutions all showed a precipitating influence upon the heated enzyme, nevertheless the inactivation temperature was either raised or left unchanged in all instances.

There is apparently little if any direct relationship between the heat inactivation and heat coagulation of emulsin under the described conditions. That inactivation of the heated enzyme occurred through a reaction involving water was demonstrated by heating the dry, powdered emulsin for half an hour to temperatures as high as 150° , without destroying its function. Heating the enzyme in absolute alcohol to the boiling point of the latter (78.4°), or in glycerol to 100° for half-hour periods did not destroy its activity as did heating to these temperatures in water.

SUMMARY.

1. Pure emulsin in dilute aqueous solutions was inactivated by incubation for 30 minutes at an upper limit of 75° , although the enzyme was not precipitated at the inactivation temperature.

2. Solutions of fluorescein and Congo red, each of which at a final concentration of 0.5 % prevents the heat coagulation of egg albumin or serum proteins, failed to advance the temperature threshold of the heat inactivation of emulsin; on the contrary, these solutions increased the sensitivity of the enzyme to heat, and lowered the inactivation temperature, as did neutral red, a colloidal dye which does not protect proteins against coagulation by heat. Very dilute solutions of Congo red (0.00025 % and below), while protecting emulsin to only a slight degree, if any, brought about a degree of mutual precipitation of enzyme and dye.

3. The action of rose Bengal upon emulsin was not determined, since this dye interfered with the hydrolysis in the controls.

4. Incubation of emulsin in solutions adjusted to various p_H values exerted significant depressing effects upon the temperature threshold of the enzyme only at p_H values as low as 1.97, and at 7.65 and above. With increasing alkalinity the enzyme was destroyed by incubation at successively lower temperatures. The rate of hydrolysis of amygdalin in the controls was not retarded until solutions at p_H 6.6 and above were employed.

5. A 0.5 % lactose solution was without appreciable influence upon the inactivation temperature of emulsin.

6. Glucose solutions from concentrations of 25 % down to 2.5 % protected emulsin somewhat against heat inactivation, the higher concentrations raising the threshold temperature several degrees. 25 % glucose exerted a slight retarding effect upon the rate of hydrolysis in the controls.

7. The emulsin was precipitated to varying degrees when incubated with crystalloidal solutes such as dilute acids, phosphate buffers or sugars.

8. With the exception of rose Bengal and the weakest of the Congo red solutions, the dyes when incubated with emulsin did not precipitate it.

9. There was apparently little if any direct relationship between the heat inactivation and heat coagulation of emulsin under the conditions of the experiments.

10. It would seem probable that emulsin forms a compound or "sorption complex" of some kind with glucose, a product of the hydrolysis of the substrate, which is capable of protecting the enzyme to a certain degree against destruction by heat.

11. Heating emulsin in the absence of water (*i.e.* in dry, powdered form to temperatures as high as 150°, in absolute alcohol to 78.4°, in glycerol to 100°) did not destroy the enzyme's function as did even lower temperatures in the presence of water.

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